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RNA interference against repulsive guidance molecule A improves axon sprout and neural function recovery of rats after MCAO/reperfusion

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ABSTRACT

Repulsive guidance molecule a (RGMa) is a neurite growth inhibitor that is of great interest in the study of CNS neuronal regeneration. We adopted RNA interference (RNAi) as a means of suppressing the expression of RGMa and observed the improvement in axonal regeneration and neurological function of rats after cerebral ischemic injury. Recombinant adenovirus rAd5-shRNA-RGMa was constructed and prepared for animal experimentation. RGMa and neurofilament protein 200 (NF200) in the ischemic cortex and ipsilateral hippocampus were detected by reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemistry. The ischemic regions were examined by triphenyltetrazolium chloride (TTC) staining and the newborn neurite branches by Biotinylated Dextran Amine (BDA) neuronal tracing. Behavior tests were adopted to evaluate neurologic function recovery. Results showed RGMa was down-regulated and axonal growth was improved in the RNAi treated group ($P<0.01$). The number of axonal sprouts of corticospinal tract from the uninjured side to the ischemic side in the RNAi treated group was increased ($P<0.01$). Behavior test scores in the RNAi treated group were significantly better than other groups after 6 weeks ($P<0.01$). RGMa in rat brains after middle cerebral artery occlusion (MCAO) can be down-regulated by RNAi successfully, which may lead to improved axonal growth and neural anatomy plasticity, as well as neuron functional recovery.

Introduction

Ischemic stroke is a severe neurological disorder that has become a great health care burden in developing countries. Rehabilitation after ischemic stroke is poor and needs to be improved. Due to the presence of numerous neurite growth inhibitors, regeneration of neuron axons is extremely difficult in the adult central nervous system (CNS) after injury (Mimura et al., 2006; Pernet et al., 2008; Schwab and Bartholdi, 1996; Winzeler AM et al., 2011). Repulsive guidance molecule a (RGMa) is a type of axonal guidance molecule that may play multiple roles in CNS development. To date, it is reported as a potential neurite growth inhibitor (Matsunaga et al., 2006; Monnier et al., 2002; Mueller et al., 2006; Schwab et al., 2005a, 2005b; Shin and Wilson, 2008). Intrathecal administration of a neutralizing antibody to block the function of RGMa can significantly enhance axonal growth and promote neurologic function recovery after spinal cord injury in rats (Schwab et al., 2005a, 2005b). Our previous studies suggested that transient middle cerebral artery occlusion (MCAO) can cause high expression of RGMa in both the ischemic cortex and ipsilateral hippocampus, accompanied with axonal depletion and motor function impairment (Zhang et al., 2011). These findings indicate that overexpression of RGMa may inhibit neurite regeneration. Suppressing RGMa may be effective for nerve regeneration and functional recovery. Therefore, in this study, RNAi was used to knock down RGMa via recombinant adenovirus.

Materials and methods

Animals

Eighty-eight healthy male adult Sprague Dawley rats were randomly assigned to 11 groups ($n=8$ in each group), including a normal group, sham group (2 d and 7 d), ischemia group (2 d and 7 d), PBS treated group (2 d and 7 d), rAd5-HK treated group (2 d and 7 d) and RNAi group (2 d and 7 d). In addition, another twenty-four rats were divided into a normal group, ischemia group, rAd5-HK treated group and RNAi group ($n=6$ in each group) to test for infarct volume and to label the corticorubral and corticospinal tracts. All protocols for

Abbreviations: DAB, 3-3′ diaminobenzidine; BDA, Biotinylated Dextran Amine; CNS, central nervous system; CCA, common carotid artery; CST, corticospinal tract; ECA, external carotid artery; GFP, Green fluorescent protein; ICA, internal carotid artery; LDF, laser-Doppler flow; MCAO, middle cerebral artery occlusion; NF-200, neurofilament protein 200; rCBF, regional cerebral blood flow; RT-PCR, reverse transcription polymerase chain reaction; RNAi, RNA interference; RGMa, repulsive guidance molecule a; TTC, triphenyltetrazolium chloride.

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animal experiments were approved by the Administrative Panel on Laboratory Animal Care of Chongqing Medical University.

Recombinant adenovirus

RGMa-specific recombinant adenovirus rAd5-shRNA-RGMa and empty carrier recombinant adenovirus rAd5-HK were supplied by the Wuhan Genesil Biotechnology Co., Ltd. The virus was amplified in human embryo kidney 293(HEK293) cells and purified by Sartorious Vivapure Adeno PACK 20. The titer was determined by 50% tissue culture infectious dose (TCID50) method (Reed and Muench, 1938).

MCAO/reperfusion in rats

Right MCAO was induced by an intraluminal filament (Zhang et al., 2008). Briefly, animals were anesthetized using 3.5% Chloral Hydrate (350 mg/kg). The body temperature was maintained at 37 °C with a homothermal blanket. Under an operating microscope, the right common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were exposed through a midline neck incision. After coagulation of the terminal artery branches of the ECA, the right ECA was coagulated. The right ICA was isolated, and the pterygopalatine artery was ligated close to its origin with a silk suture. Two microaneurysm clips were placed across both the CCA and ICA to block blood flow temporarily during the insertion of the suture. A small incision was made on the ECA stump, and a 40 mm-length monofilament nylon suture, heat blunted at the tip and coated with melted paraffin wax, was inserted into the ICA. After removing the clip on the ICA, the nylon suture was advanced 18–20 mm from the bifurcation of the CCA until mild resistance was felt, then it was tightened by a silk suture around the ECA stump. Another microaneurysm clip was removed, and the neck incision was closed. After 2 h of occlusion, the animals were re-anesthetized and the filament was withdrawn. Animals in the sham group were treated similarly, except that the filament was not advanced to the origin of the MCA. The rats without neurological deficit or with subarachnoid hemorrhage after reperfusion were excluded from this study. Physiological parameters were monitored before, during and after MCAO. Regional cerebral blood flow (rCBF) was measured during the surgery by a laser-Doppler flow (LDF) (Periflux system 5000; Perimed) to confirm the successful occlusion of MCA.

Stereotactic surgery

Immediately after reperfusion, rats were placed in a stereotaxic apparatus. Three different titers of rAd5-shRNA-RGMa and PBS were injected into the ischemic cortex and ipsilateral hippocampus of rats ($n = 32$) after MCAO/reperfusion by stereotactic surgery. The bregma was used as stereotaxic zero. Two sites on the right cortex surrounding the infarction (Zhao et al., 2002) were targeted for injection at the following coordinates: 1.0 mm rostral to the bregma, 2.0 mm lateral to the midline, 1.2 mm ventral to the dura; 3.0 mm caudal to the bregma, 1.5 mm lateral to the midline and 1.2 mm ventral to the dura. The coordinates on the ipsilateral hippocampus were: 3.5 mm caudal to the bregma, 2.5 mm lateral to the midline and 3.5 mm ventral to the dura. The injection rate was 0.3 μl/min and the total volume was 2 μl for each site. At the end of the injection, the microinjector was kept immobile for 5 min before withdrawal.

Detection of virus delivery

Green fluorescent protein (GFP) was observed under fluorescence microscope for detection of virus delivery. The transfection efficiency was calculated as the ratio of GFP positive cells to total cells. To determine the proper therapeutic titer, pathological changes and inflammation around the injection sites were examined by HE staining and immunohistochemistry of IL-1β. The most appropriate therapeutic titer was used in the following study.

Infarct volume

Infarct volume was determined by 2, 3, 5-triphenyltetrazolium chloride (TTC) staining (+4.7 to −5.5 mm from the bregma) (Paxinos and Watson, 1986). Using a rat brain slicer (Activational Systems, Warren, MI, USA), 2 mm coronal sections were made. Slices were then immersed in 2% TTC at 37 °C for 30 min, photographed, and fixed in 4% paraformaldehyde for 24 h. The infarct size was measured as described by Kawamata et al. (1997).

Immunohistochemistry

At 2 d and 7 d, rats were deeply anesthetized and fixed through the left ventricle by first perfusing PBS, then 4% paraformaldehyde.

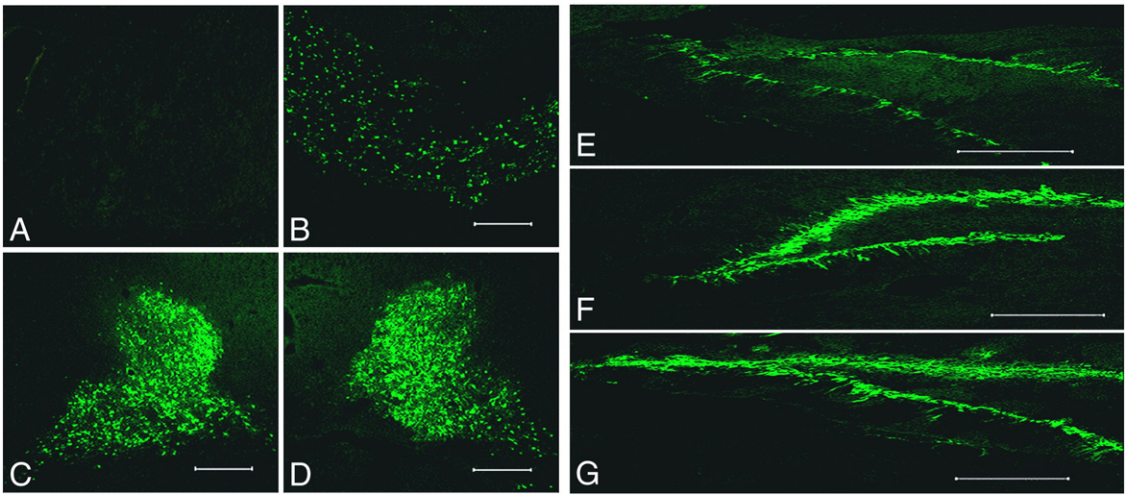

Fig. 1. Adenovirus delivery on peri-infarct cortex and ipsilateral hippocampus 7 days after injection was observed using Laser Confocal Scanning Microscope (×100). PBS and three different titers of rAd5-shRNA-RGMa were injected into the ischemic cortex and ipsilateral hippocampus after MCAO/reperfusion by stereotactic surgery. A: PBS-injected cortex had no GFP positive cells. B: A few GFP positive cells were localized in the ischemic cortex injected with low titer (5.01×10^9 pfu/ml) of adenovirus. C, D: A large number of GFP positive cells were observed in the ischemic cortex injected with medium (2.51×10^{10} pfu/ml) and high titer (5.01×10^{10} pfu/ml) adenovirus. E, F, and G: The ipsilateral hippocampi were injected with the low, medium and high titers of adenovirus respectively. All three titers of rAd5-shRNA-RGMa were able to transfect the ischemic cortex and ipsilateral hippocampus in rats' brains. Scale bars: 300 μm.

The brains were removed and post-fixed in 4% paraformaldehyde for 24 h (4 °C). Ten micron serial sections embedded in paraffin were prepared for histological and immunohistochemical analysis. Sections were deparaffinated, rehydrated, incubated in 3% hydrogen peroxide (H_2O_2) for 10 min, then heated in the microwave in a citrate buffer (pH 6.0) for antigen retrieval. After blocking in normal serum for 30 min at 37 °C, the sections were incubated with various primary antibodies (rabbit anti-RGMa, 1:100, Abcam; mouse anti-Neurofilament 200, 1:100, Santa Cruz Inc.; rabbit anti-IL-1β, 1:100, Zhongshan Goldenbridge Biotechnology CO., Ltd.) overnight at 4 °C. After rinsing with PBS, the sections were incubated with either goat anti-rabbit IgG or goat anti-mouse IgG for 2 h. Positive activity was revealed by 3-3′ diaminobenzidine (DAB). Protein expression levels were reflected by the mean optical density value.

RT-PCR for RGMa mRNA

The procedure was similar to that in our previous study (Wang et al., 2010). The primer sequences were designed as follo0ws: sense-5′ CGT AAA GAC CTC TAT GCC AAC A-3′, antisense-5′ CGG ACT CAT CGT ACT CCT GCT-3′ (β-actin, product size 229 bp); sense-5′ GCT GGA TGG ATG GGT ATG GG-3′, antisense-5′ GCC GCA GTG AGT GTA GTT GG-3′ (RGMa, product size 456 bp). The amplification program consists of a denaturing step at 94 °C for 2 min, an annealing step at 59.3 °C for 50 s and an extension step at 72 °C for 2 min for 35 cycles. The 5 μl PCR products were separated on a 2% agarose gel (E-Gel, Invitrogen, Carlsbad, CA, USA). The gel was photographed under UV transillumination with an Alpha Imager system (AlphaInnotech, San Leandro, CA, USA). RGMa mRNA expression levels of RGMa were represent by the band density relative to that of β-actin.

Evaluation of sensorimotor performance

Rats were trained for food grab for 1 week. Montoya's staircase test (Montoya et al., 1991) was carried out 1 d before and 7 d, 2 weeks and 6 weeks after MCAO/reperfusion to test skilled forepaw use. The procedure was as follows: All animals were food restricted to 85–90% of their free feeding weight one day before the test. On the next day, rats were

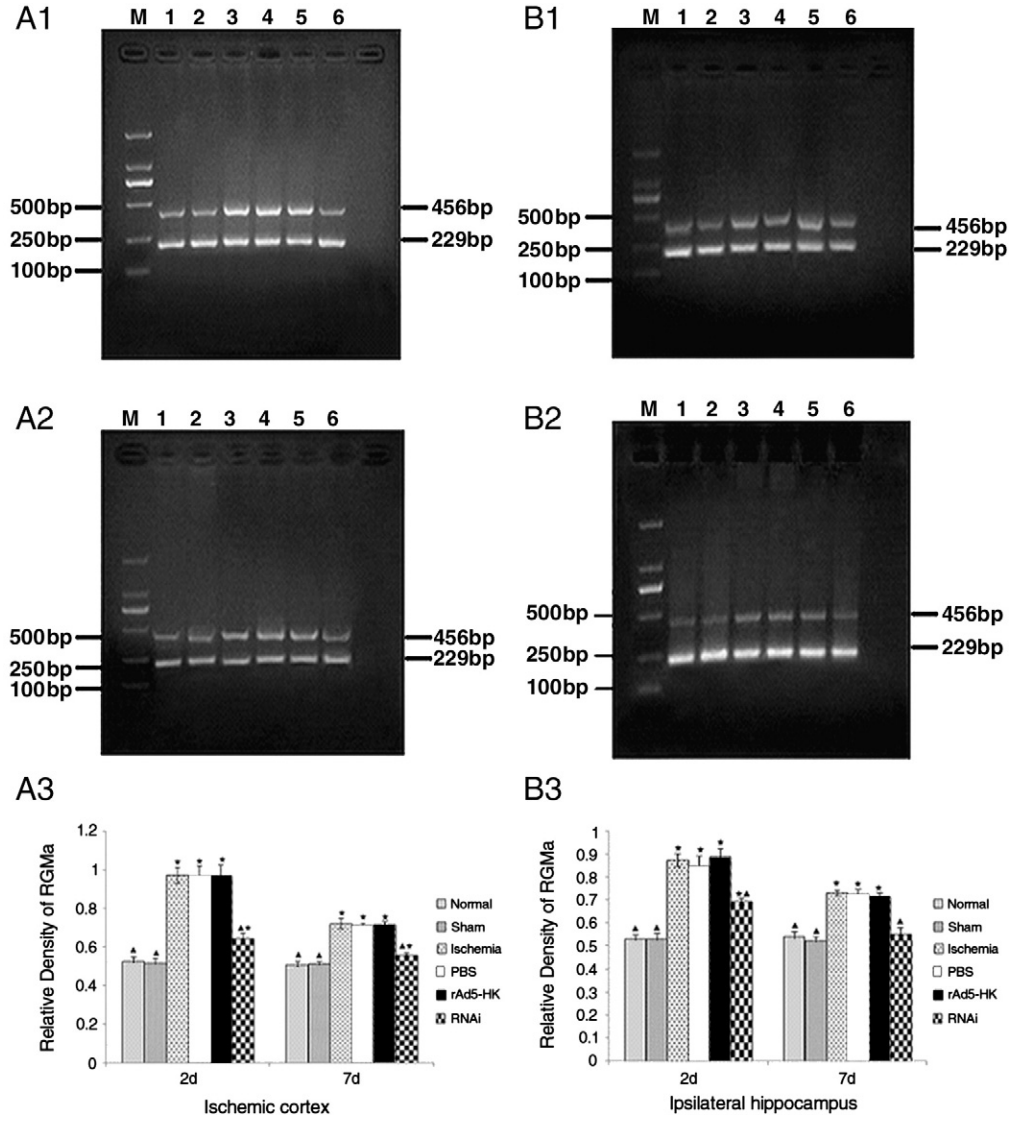

Fig. 2. RT-PCR analysis demonstrates the expressions of RGMa mRNA in the ischemic cortex (A1, A2) and ipsilateral hippocampus (B1, B2) 2 days and 7 days after MCAO/reperfusion. M: Marker; 1: Normal group; 2: Sham group; 3: Ischemia group; 4: PBS group; 5: rAd5-HK groups; 6: RNAi group. At 2 days, the RGMa mRNA of ischemia, PBS and rAd5-HK groups in the ischemic cortex (A1) and ipsilateral hippocampus (B1) were greatly increased, while RNAi treatment was able to significantly inhibit the gene expression of RGMa (A3, B3). Although expression decreased gradually over one week (A2, B2), the RGMa mRNA in ischemia, PBS and rAd5-HK groups was still higher than that of RNAi group. Bars represent the relative density of RGMa to β-actin in the ischemic cortex (left) and ipsilateral hippocampus (right). Results are expressed as mean ± SD. ★$P<0.01$, compared to normal group at the same time point; ▲$P<0.01$, compared to ischemia group at the same time point.

placed in a Plexiglas box with six steps bilaterally and a round feeding trough on each step. Two chow pellets (45 mg) were placed only on the troughs on the left, and the pellets were replenished every 5 min. The number of pellets seized or eaten in 15 min was used as a measure of forelimb reaching ability.

Biotinylated dextran amine (BDA) tracing

Four weeks after MCAO, the left sensorimotor cortex was exposed and 5 injections (1 μl) of 10% solution of BDA (MW10,000, Molecular Probes, CA, U.S.A.) were injected following these five coordinates: 1.0 mm rostral to the bregma, 1.0 mm lateral to the midline, 1.5 mm ventral to the dura; 4.0 mm caudal to the bregma, 1.0 mm lateral to the midline, 1.5 mm ventral to the dura; 1.0 mm rostral to the bregma, 5.0 mm lateral to the midline, 1.5 mm ventral to the dura; 4.0 mm caudal to the bregma, 5.0 mm lateral to the midline, 1.5 mm ventral to the dura; 1.5 mm caudal to the bregma, 2.5 mm lateral to the midline, 1.5 mm ventral to the dura. Two weeks after the BDA injection, animals were perfused cardially. The brain was dissected, post-fixed overnight and embedded in tissue-freezing medium for cryostat sectioning. Forty micrometer coronal sections were made using a cryostat microtome (Leica CM3050S, Germany). All sections were collected in 0.01 mmol/l PBS and incubated with avidin-peroxidase in 0.01 mmol/l PBS/0.3% Triton X-l00 for 4 h at 37 °C. The tissue was incubated with 0.05% DAB (Molecular Probes, CA, USA) for 5 min.

The brain structures that needed to be analyzed were identified using the anatomy atlas by Paxinos and Watson (1986). Sections were analyzed using an image analysis software (MCID/M2-Analyzing Program, Imaging Research, Ontario, Canada). The number of labeled corticospinal tract (CST) fibers in the left pons was counted and used for error correction in BDA labeling. Images of two consecutive sections at the level of the cerebral peduncle were captured using a 10× objective. Then four squares, each measuring 0.45×0.67 mm, were centered on the cerebral peduncle and the BDA-positive fibers within these squares were examined using a 40× objective. The total number of labeled CST fibers for each section was estimated using the means of the four values. The values obtained from the two consecutive sections were averaged. The corticorubral fibers projecting to the right red nucleus were examined by counting all BDA-positive fibers crossing the midline on each section. To account for differences in tracing, the number of midline-crossing BDA-positive fibers was divided by the total number of CST fibers.

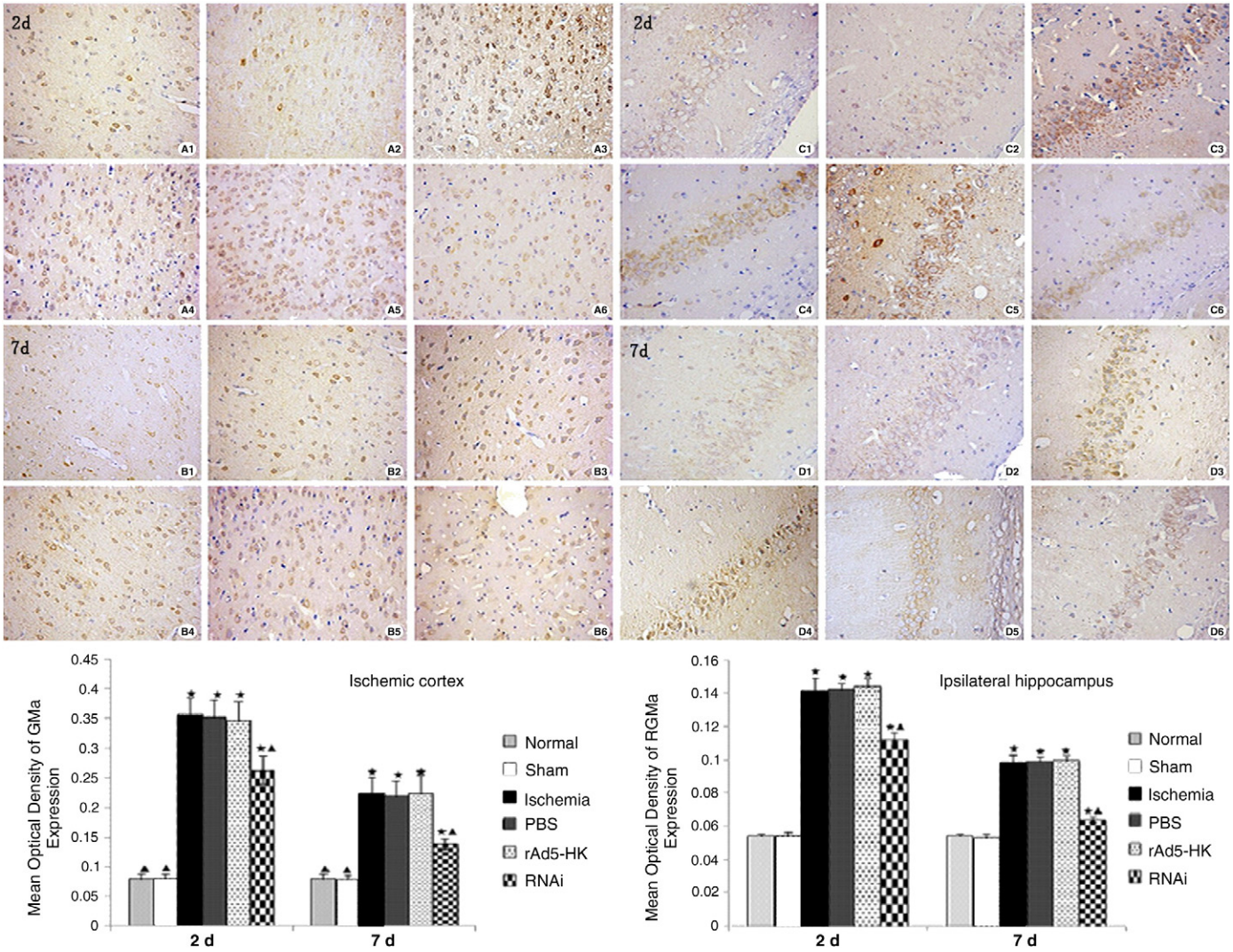

Fig. 3. RGMa expression in ischemic cortex and ipsilateral hippocampus was detected by immunohistochemistry 2 days and 7 days after MCAO/reperfusion. 1: Normal group; 2: Sham group; 3: Ischemia group; 4: PBS group; 5: rAd5-HK groups; 6: RNAi group. In the cortex, the RGMa protein was highly expressed 2 days after MCAO/reperfusion (A3–A5) but decreased a little by 7 days (B3–B5). The protein in rats treated with adenovirus (A6, B6) was significantly decreased at 2 days and 7 days in comparison with that of ischemia, PBS and rAd5-HK groups. The changes in the ipsilateral hippocampus were similar to those of the cortex (C3–C5, D3–D5). RNAi treatment (C6, D6) could suppress the overexpression of RGMa protein after ischemia. Bars represent the mean optical density of RGMa in the ischemic cortex (left) and ipsilateral hippocampus (right). Results are expressed as mean ± SD. ★$P<0.01$, compared to normal group at the same time point; ▲$P<0.01$, compared to ischemia group at the same time point.

Statistical analysis

Statistical analysis was performed using SPSS 12.0 for windows. All results were presented as means ± standard deviation (SD). Statistical differences between the control and each group of ischemia (with or without adenovirus treatment) were compared using one-way analysis of variance (ANOVA) followed by a post hoc Tukey test. A P-value <0.05 was considered statistically significant.

Results

Titers, delivery efficacy and toxicity of recombinant adenovirus

The titer of rAd5-HK used was 3.16×10^{10} pfu/ml. The titers of rAd5-shRNA-RGMa used were 5.01×10^{9} pfu/ml (low titer), 2.51×10^{10} pfu/ml (medium titer), and 5.01×10^{10} pfu/ml (high titer). All three titers of rAd5-shRNA-RGMa were able to successfully transfect the ischemic cortex and ipsilateral hippocampus in rats' brains (Fig. 1). Aside from the injected cortex and hippocampus, GFP positive cells are also be found in the corpus callosum, lateral ventricle and choroid plexus. The transfection efficiency of medium (68.63 ± 9.58%) and high titer injections (69.51 ± 9.36%) were similar (P > 0.05), and both were better than the efficiency of the low titer group (27.35 ± 7.92%, P < 0.01, Fig. 1A).

The phenomenon of perivascular inflammatory cell infiltration was only observed around the injection site in brain tissues in the high titer group (Fig. 1B). IL-1β was increased 2 days after injection (PBS control, low titer, medium titer and high titer groups, compared to normal group, P < 0.01). IL-1β in the PBS control, low or medium titer group returned to normal 7 days after adenovirus injection (compared to normal group, P > 0.05, Figs. 1C, B–D). Only the high titer group showed a significantly higher level of IL-1β expression compared to the normal group (P < 0.01, Figs. 1C, E). Those results together suggested that the medium titer of rAd5-shRNA-RGMa was proper for treatment use.

RGMa mRNA in ischemic cortex and ipsilateral hippocampus

The expression of RGMa mRNA in both the cortex and hippocampus of the ischemia group (2 d and 7 d) was higher than that in the control and sham groups (P < 0.01). RNAi treatment was able to significantly decrease (P < 0.01) the high expression of RGMa mRNA in the ischemic cortex and ipsilateral hippocampus after 48 h, and the levels returned to nearly normal on 7 d (Fig. 2).

RGMa protein in ischemic cortex and ipsilateral hippocampus

The results were similar to those of RGMa mRNA expression. The protein was highly expressed in both the ischemic cortex and the ipsilateral hippocampus after MCAO/reperfusion (P < 0.01). Gene suppression using RNAi was able to inhibit over expression of RGMa protein in the ischemic cortex and hippocampus on day 2 and day 7 (P < 0.01, Fig. 3).

Detection of infarct volume

Infarct volume was detected by TTC staining 2 days after MCAO. No significant differences were found between the MCAO/reperfusion (243.19 ± 9.81 mm^3), negative control (238.90 ± 7.29 mm^3) and RNAi groups (235.46 ± 11.26 mm^3) (P > 0.05). This indicates that the treatment failed to reduce the infarct volume (Fig. 4).

Axon growth

The axons in rat cortex and hippocampus of the normal and sham groups were neatly aligned, with long NF-200 positive fibers. The axons were disorderly and became shorter and less dense 2 days after ischemia/reperfusion. The recovery at 7 days was poor. RNAi treatment was able to improve the axon growth after ischemic injury. Axons in adenovirus treated rats showed an early regeneration 2 days after ischemia, and the recovery was obvious at 7 days (P < 0.01, Fig. 5).

Tracing corticorubral and corticospinal tracts

Pyramidal cells and astrocytes in the left cortex sensorimotor area can be observed in rats in each group. Meanwhile the neural fibers extending from left to right were labeled by BDA. In the normal control group, a very small number of corticorubral fibers (17.27 ± 9.77) crossed to the right side; more corticorubral fibers in the ischemia group (92.58 ± 20.78), rAd5-HK treated group (84.61 ± 24.94) and RNAi group (203.75 ± 33.12) sprouted lateral branches, formed dense fiber bundles and crossed the midline to reach the red nucleus in the midbrain. The number of corticorubral fibers crossing the midline in the RNAi group was much higher than in the other two groups (P < 0.01, Fig. 6).

Neural function evaluation

The ability of food grab by rats using their left forelimbs showed no significant difference in each group before operation (Montoya's staircase test scores in normal group: 11.00 ± 1.41; ischemia group: 11.00 ± 1.26; rAd5-HK treated group: 10.83 ± 1.17; RNAi group: 10.83 ± 1.47, P > 0.05). The scores of the ischemia group (3.17 ± 1.17) and rAd5-HK treated group (3.50 ± 1.05) decreased to 25% of the normal level 7 d after operation, while the RNAi group was above 38% (4.83 ± 1.47) of the normal. In the following five weeks, the scores in

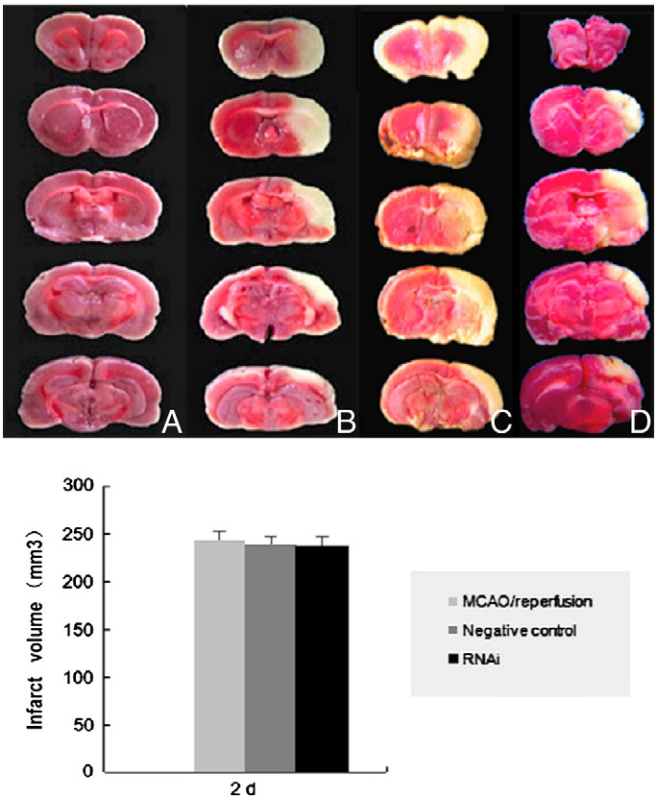

Fig. 4. The infarct areas of rat brains were stained by TTC 2 days after ischemia. A: Normal group; B: Ischemia group; C: rAd5-HK groups; D: RNAi group. In these pictures, the white parts of the brains were ischemic tissue that could not be stained by TTC. Comparison of the total volume of each infarct brain found no difference among the four groups, suggesting that RNAi treatment was not favorable in reducing infarct volumes. Bars represent the infarct volumes in the four groups. Results are expressed as mean ± SD.

the ischemia and rAd5-HK groups were increased slowly compared to the RNAi group. After 2 weeks, the scores rose to 40% in the ischemia (5.33 ± 1.03) and rAd5-HK treated groups (6.67 ± 1.63), while the RNAi group increased to 60% (8.50 ± 1.04) of normal. At 6 weeks, the RNAi group demonstrated a 90% functional recovery (9.33 ± 1.37) of left forelimb as compared to the ischemia (6.83 ± 1.17) and rAd5-HK treated groups (7.83 ± 1.72, $P<0.01$, Fig. 7).

Discussion

The recombinant adenoviral vector is particularly useful for gene therapy in CNS injury because of its characteristics such as a short life span, unconformity to host genomes, safety and stability (He et al., 1998; Lim et al., 2010; Mahanivong et al., 2006; Shyu et al., 2005). However, a large dose of shRNA will disrupt the micro RNA pathway in vivo, resulting in mouse death (Grimm et al., 2006). The recombinant adenovirus itself and its gene products both have immunogenic effects, which means its transduction into living tissues or cells might provoke an immune response (Lowenstein et al., 2007; Okamba et al., 2007; Pichla-Gollon et al., 2009; Zaiss et al., 2009) that may influence its treatment effects. To avoid these side effects, local inflammatory cell infiltration at the injection site and the expression of IL-1, a marker of early inflammatory response to adenovirus (Cartmell et al., 1999), was evaluated by pathological staining and immunohistochemistry. The efficacy of the adenovirus to transfect neurons in vivo must also be taken into account. Thus, the inflammation intensity and transfection efficacy of three titers of adenovirus were compared to determine the best titer for the study. Since the medium titer group showed higher transfection efficiency than the low titer group and lower inflammatory infiltrations side-effects than the high titer group, it was most appropriate titer for treatment.

In the adenovirus group, both the rat cortex mRNA and the RGMa protein were significantly decreased 2 d after MCAO compared to the ischemic rat, PBS treated rat and rAd5-HK treated rat. At day 7, the RGMa was closest to normal, indicating the high efficiency of gene silencing by RNAi. Although MCAO cannot directly cause hippocampus infarction, neurons in the hippocampal CA1 are sensitive to ischemia, and widespread death appears 48–72 h after ischemia. This phenomenon is termed delayed neuronal death (Sopala et al., 2000). In addition, in our previous work, we found RGMa changes in the

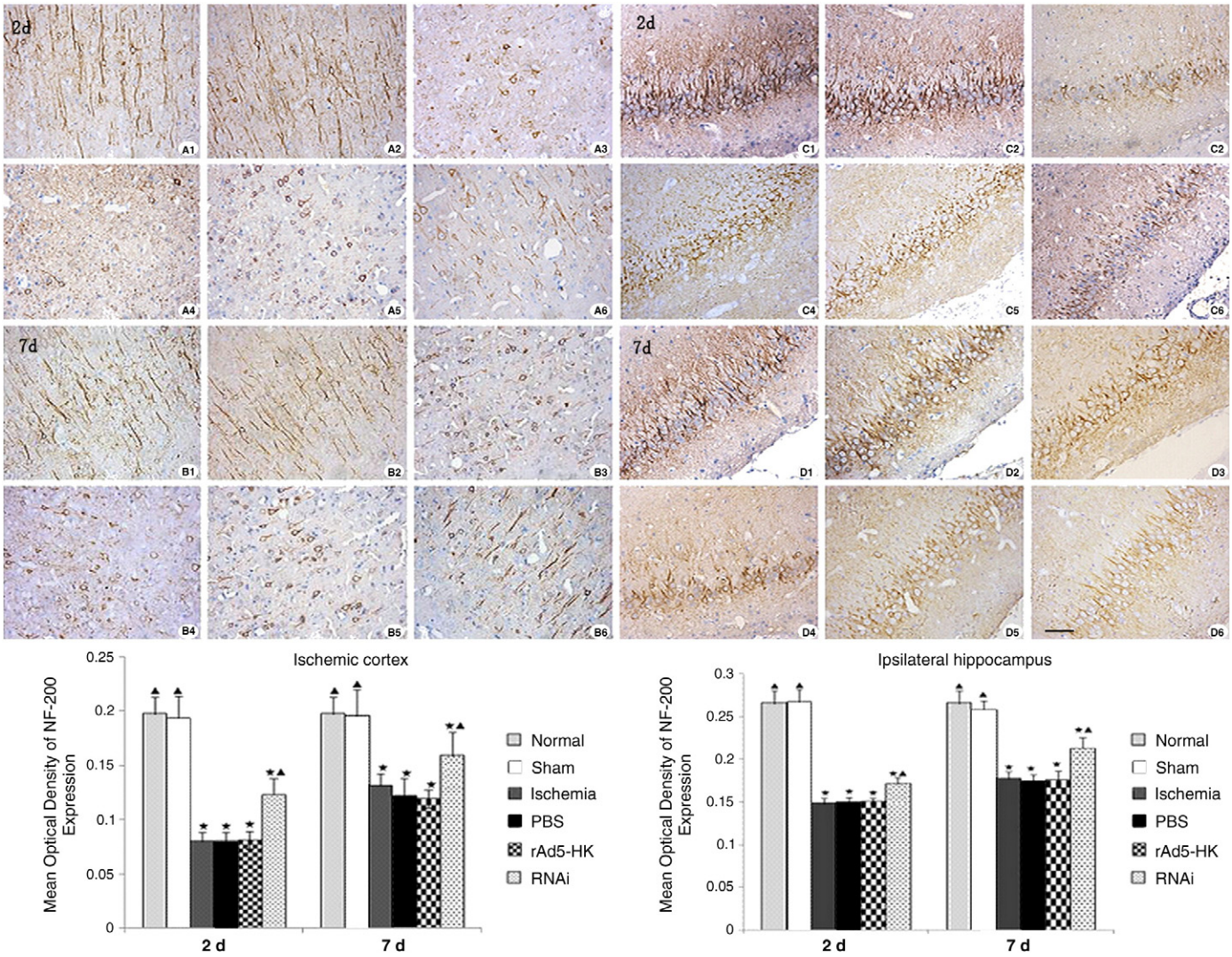

Fig. 5. NF-200 protein indicating the axonal ischemic injury was investigated in ischemic cortex (A1–A6, B1–B6) and ipsilateral hippocampus (C1–C6, D1–D6) 2 days and 7 days after MCAO/reperfusion. 1: Normal group; 2: Sham group; 3: Ischemia group; 4: PBS group; 5: rAd5-HK groups; 6: RNAi group. In non-ischemic cortex (A1, A2, B1, B2), axons were long and neatly arranged. The cortical axons were seriously damaged by ischemia at 2 days (A3–A5), and the recovery at 7 days (B3–B5) was poor. Only axons in adenovirus treated rats (A6) showed an early regeneration after ischemia, and the recovery was obvious at 7 days (B6). The damage to hippocampal axons was not as severe as in the cortex (C3–C5) and recovery was good (D3–D5). The effect of RNAi treatment on hippocampal axons (C6, D6) was similar to that in the cortex. Bars represent the mean optical density of NF-200 in the ischemic cortex (left) and ipsilateral hippocampus (right). Results are expressed as mean ± SD. ★$P<0.01$, compared to the normal group at the same time point; ▲$P<0.01$, compared to the ischemia group at the same time point. Scale bars: 100 μm.

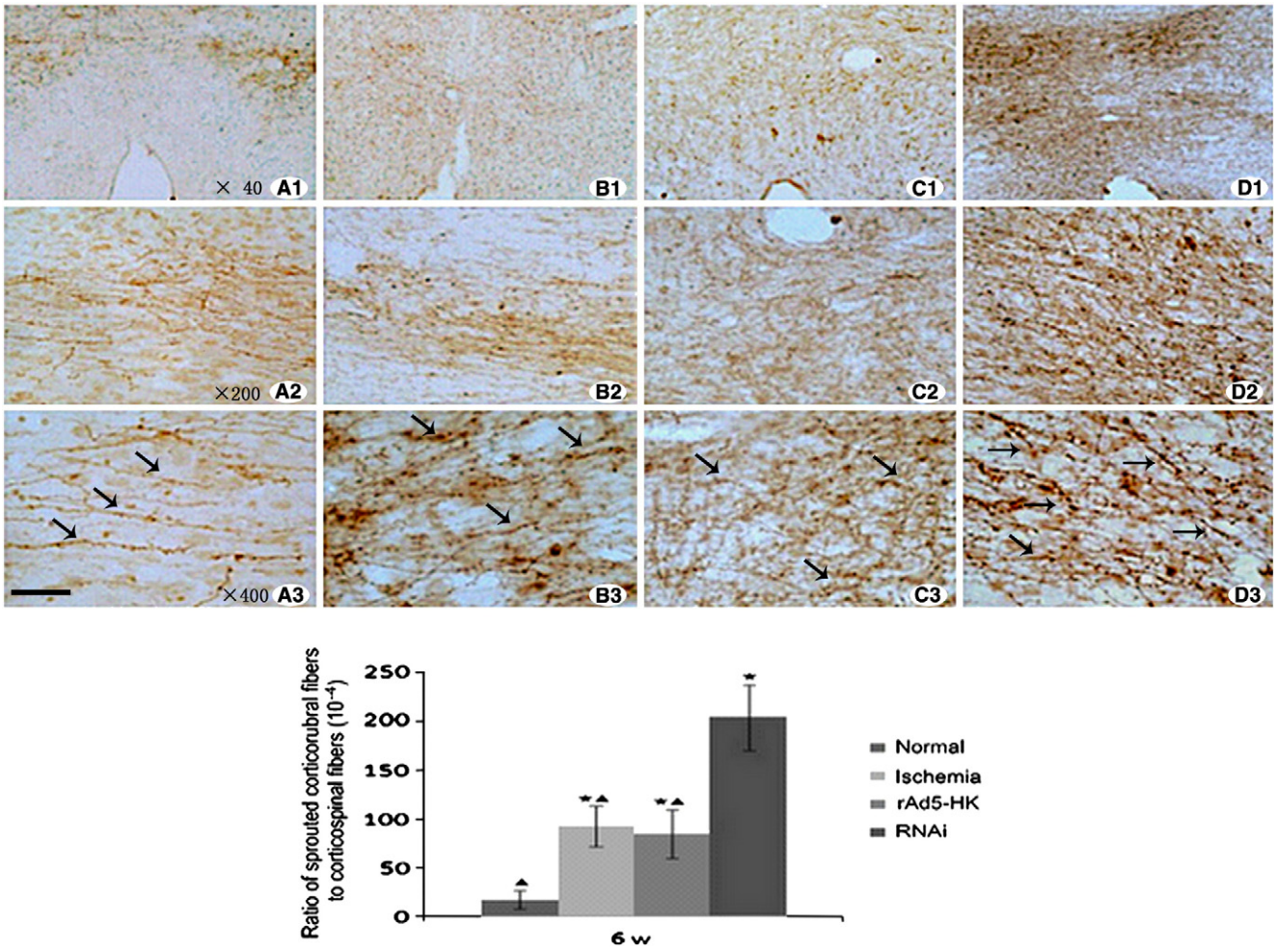

Fig. 6. Contralateral corticorubral fibers crossing midline to the right red nucleus were labeled using BDA 6 weeks post-operation. A: Normal group; B: Ischemia group; C: rAd5-HK group; D: RNAi group. 1: ×40; 2: ×200; 3: ×400. In midbrain, a small number of corticorubral fibers crossed from the left side to the right side in normal conditions (A1–A3). After ischemia, more transverse fibers were labeled around the midline, but these fibers were limited in length (B1–B3, C1–C3). With RNAi treatment, the limitation of fiber length was mostly ameliorated, as a large number of fibers were observed around the right red nucleus (D1–D3). Bars represent the ratio of sprouted corticorubral fibers to corticospinal fibers crossing midline in the four groups. Results are expressed as mean ± SD. Arrows indicate the fibers crossing the midline. ★P<0.01, compared to the normal group; ▲P<0.01, compared to the RNAi intervention group. Scale bars: 20 μm.

hippocampus after ischemia (Zhang et al., 2011). Therefore, it is important to observe the change of RGMa in the hippocampus. We injected adenovirus directly into it and found that the ipsilateral hippocampus followed a similar pattern of RGMa expression as the ischemic cortex. Meanwhile, we found that the regeneration of injured axons including the number, arrangement and the length of NF-200 positive fibers was improved at both time points (2 d, 7 d) by RNAi treatment. Down-regulation of RGMa was accompanied by repair of axons both spatially and temporally. Our findings were consistent with the results of RGMa intervention strategy in other brain trauma and spinal cord injury animal models (Hata et al., 2006; Schwab et al., 2005a, 2005b).

Infarction occurred in rat brains 2 days after the right middle cerebral artery occlusion operation. However, RNAi treatment failed to reduce the infarct volume. Considering the positive effects of RGMa suppression on axon growth, it is inferred that RNAi treatment may play a role in attenuation of axonal injury by inhibiting the overexpression of RGMa in lesions rather than neuro-protection. The underlying mechanisms might lie in axonal connectivity in the corticorubral pathways, which constitutes the anatomical substrate responsible for recovery of motor function (Andres et al., 2011; Kartje et al., 1999; Papadopoulos et al., 2002). In RNAi treated animals, many BDA positive fibers of the contralateral corticorubral tract sprouted branches to cross the midline to the ischemic side. This suggests that reducing the over-expressed RGMa in the ischemic cortex favors the crossing of the healthy neural fibers from the contralateral cortex, which is in accordance with the manner of axonal compensation to synaptic contacts (Dickson et al., 2007; Fenrich et

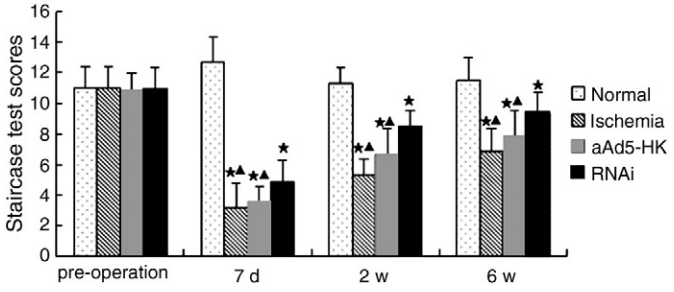

Fig. 7. The rat left forelimb motor function was evaluated using the Staircase test. Bars represent Staircase test scores in the four groups. Before the operation, the scores of rats did not differ significantly in each group. The scores in the ischemia and rAd5-HK groups were decreased to nearly one quarter of the normal 7 days post-operation, while the RNAi group was above 38% of the normal. After 2 weeks, the scores rose to 40% of normal in the ischemia and rAd5-HK treated groups, while the RNAi group increased to 60% of normal. At 6 weeks, the RNAi group demonstrated a 90% functional recovery of left forelimb as compared to the ischemia group and rAd5-HK treated group (P<0.01). Results are expressed as mean ± SD. ★P<0.01, compared to the normal group; ▲P<0.01, compared to RNAi intervention group at the same time post-operation.

al., 2007). The results of the staircase test indicated that ischemia induced a 75% reduction of the left forepaw motor function 7 days after MCAO surgery. Although some dysfunction remained in non-RNAi treated rats, the motor function was greatly improved by RNAi treatment at 2 weeks and almost completely recovered at 6 weeks.

Generally speaking, suppression of RGMa by RNAi via recombinant adenovirus can enhance the axonal sprouting and functional recovery in the rat experimental stroke model. RNAi may be a potential gene therapy for ischemic stroke patients. At present, the RGMa/RhoA signaling pathway is controversial, and will be studied further in our future research.

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Disclosure/conflict of interest

The authors declare that they have no conflict of interest.

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